Environ. Sci. Technol. **2002**, *36,* 3787−3796

^{15}N NMR Investigation of the Covalent Binding of Reduced TNT Amines to Soil Humic Acid, Model Compounds, and Lignocellulose

K. A. THORN* AND K. R. KENNEDY

U.S. Geological Survey, P. O. Box 25046 M.S. 408, Denver Federal Center, Denver, Colorado 80225-0046

The five major reductive degradation products of TNT—4ADNT (4-amino-2,6-dinitrotoluene), 2ADNT (2-amino-4,6-dinitrotoluene), 2,4DANT (2,4-diamino-6-nitrotoluene), 2,6DANT (2,6-diamino-4-nitrotoluene), and TAT (2,4,6-triaminotoluene)—labeled with ^{15}N in the amine positions, were reacted with the IHSS soil humic acid and analyzed by ^{15}N NMR spectrometry. In the absence of catalysts, all five amines underwent nucleophilic addition reactions with quinone and other carbonyl groups in the soil humic acid to form both heterocyclic and nonheterocyclic condensation products. Imine formation via 1,2-addition of the amines to quinone groups in the soil humic acid was significant with the diamines and TAT but not the monoamines. Horseradish peroxidase (HRP) catalyzed an increase in the incorporation of all five amines into the humic acid. In the case of the diamines and TAT, HRP also shifted the binding away from heterocyclic condensation product toward imine formation. A comparison of quantitative liquid phase with solid-state CP/MAS ^{15}N NMR indicated that the CP experiment underestimated imine and heterocyclic nitrogens in humic acid, even with contact times optimal for observation of these nitrogens. Covalent binding of the mono- and diamines to 4-methylcatechol, the HRP catalyzed condensation of 4ADNT and 2,4DANT to coniferyl alcohol, and the binding of 2,4DANT to lignocellulose with and without birnessite were also examined.

Introduction

Contamination of soils by the explosive 2,4,6-trinitrotoluene (TNT) on military bases and former munitions manufacturing plants is a worldwide problem (*1*−*5*). Because leaching of TNT and its toxic reductive degradation products into ground and surface waters is a major health concern, regulators have mandated cleanup of contaminated soils. The number of sites designated for remediation in the United States alone has been conservatively estimated at 1500 (*6*).

In the past, wastewater streams containing TNT and RDX generated during the manufacture, packing, and decommissioning of outdated munitions were directed to lagoons for primary settling of solid munitions material before the water was released to streams and rivers. Evaporation of the lagoons left areas, typically a few acres in size, heavily contaminated with the parent explosives and their degradation products (*5*). Soils have also been contaminated at open burning/open detonation areas and firing ranges (*6*); the distribution of contaminants on these sites can be extremely heterogeneous (*7*). Among biological treatments for contaminated soils that can be excavated and remediated in a nearby facility, windrow composting has been implemented at several sites within the United States (*3, 8*). For example, at the Umatilla Army Depot in Hermiston, OR, windrow composting of 20 000 tons of washout lagoon soils containing 1563 ppm TNT, 953 ppm RDX, and 156 ppm HMX reduced contaminant levels to 4 ppm TNT, 2 ppm RDX, and 5 ppm HMX (*9, 10*).

During composting, and under natural conditions in soils and sediments, TNT undergoes reduction of one or more nitro groups via nitroso and hydroxlamino intermediates (Figure 1) to form the monoamines 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT), and the diamines 2,4-diamino-6-nitrotoluene (2,4DANT) and 2,6-diamino-4-nitrotoluene (2,6DANT) (*11*−*15*). Under strictly anaerobic conditions (E_h < −200 mV), complete reduction to 2,4,6-triaminotoluene (TAT) may occur. A number of reports have established that the amines do not undergo mineralization but form solvent nonextractable bound residues with organic matter (*11, 12, 16*−*18*). The ability to partially release the solvent nonextractable amines through acid and base hydrolyses has lead researchers to conclude that the amines form covalent bonds with organic matter. The amines are considered detoxified once covalently bonded to the organic matter of soil or compost.

The environmental safety of composting assumes that the potential for re-release of the aromatic amines from treated soils over the long term is negligible. Confirmation of covalent binding and a determination of the types of bonds formed between the amines and organic matter is therefore critical to an overall assessment of composting as a remediation strategy. Recent ^{15}N NMR studies provided direct spectroscopic evidence for the covalent binding of aniline, the parent compound of aromatic amines, to humic substances, to model compounds representing functional groups present in humic substances, and to the organic matter of whole soil and peat (*19, 20*). In this paper, covalent binding to organic matter by the five major reduced TNT amines (4ADNT; 2ADNT; 2,4DANT; 2,6DANT; TAT), all labeled with ^{15}N in the amine positions, is examined in detail. The mono- and diamines were reacted with 4-methylcatechol under noncatalyzed conditions. The horseradish peroxidase (HRP) catalyzed self-condensation reactions of 2,4DANT and 2,6DANT, and the HRP catalyzed condensation of 4ADNT and 2,4DANT with coniferyl alcohol were also examined. All five amines were reacted with the IHSS soil humic acid in the presence and absence of HRP, and in the case of 2,-4DANT, also with birnessite as catalyst. Sawdust (lignocellulose) was reacted with 2,4DANT alone and with birnessite as catalyst. The objective is to confirm covalent binding of all the amines and investigate differences in reactivity of the aromatic amines with organic matter. A knowledge of the types of bonds formed between the individual amines and organic matter is a prerequisite for the study on the transformation of T^{15}NT in an aerobic compost described in the companion paper (*21*) and for an understanding of bioremediation and natural attenuation processes in general.

Experimental Section

Materials. The soil reference humic acid (SHA; from Elliot silt loam soil, Joliet, IL) was purchased from the International Humic Substances Society (IHSS). The labeled compounds 4-amino-^{15}N-2,6-dinitrotoluene (4ADNT), 2-amino-^{15}N-4,6-dinitrotoluene (2ADNT), and 2,4,6-triamino-$^{15}N_3$-toluene

* Corresponding phone: (303)236-3979; fax: (303)236-3934; e-mail: kathorn@usgs.gov.

10.1021/es011383j Not subject to U.S. Copyright. Publ. 2002 Am. Chem. Soc.
Published on Web 08/06/2002

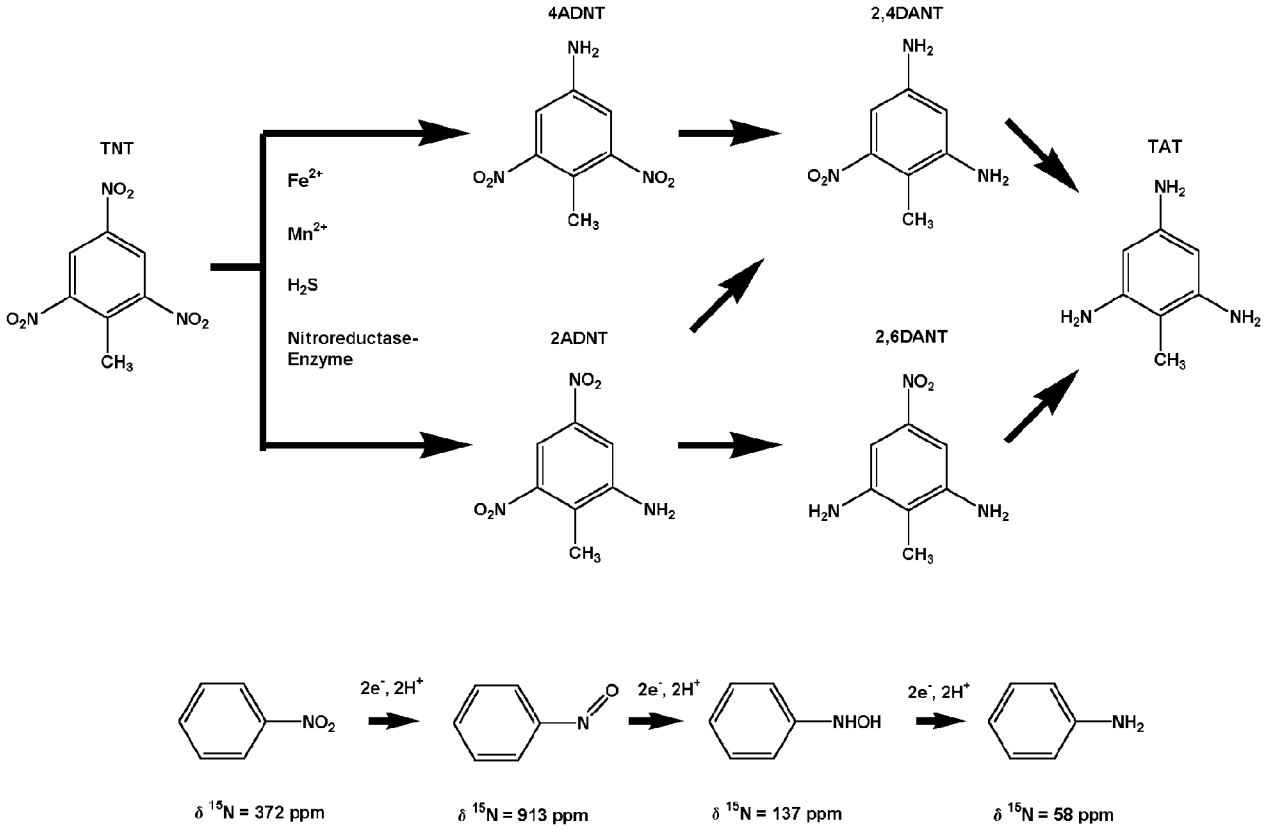


FIGURE 1. Reduction of TNT to aromatic amines. Reduction potentials have been reported by Hofstetter et al. (*13*). In composting environments, reduction is performed by the bacterial and fungal consortia through the action of nitroreductase enzymes (*14*). Ferrous iron associated with iron(III)(hydr)oxide surfaces is considered the dominant reductant in anaerobic aquifers (*15*). 2ADNT = 2-amino-4,6-dinitrotoluene; 4ADNT = 4-amino-2,6-dinitrotoluene; 2,4DANT = 2,4-diamino-6-nitrotoluene; 2,6DANT = 2,6-diamino-4-nitrotoluene; TAT = 2,4,6-triaminotoluene

trihydrochloride (TAT) were custom synthesized by Dr. Ron Spanggord, SRI International, Menlo Park, CA (*22*). The labeled diamines 2,4-diamino-$^{15}N_2$-6-nitrotoluene (2,4DANT) and 2,6-diamino-$^{15}N_2$-4-nitrotoluene (2,6DANT) were purchased from ISOTEC. Coniferyl alcohol and 4-methylcatechol were purchased from Aldrich. Birnessite and HRP were described previously (*19*). (Use of trade names in this report is for identification purposes only and does not constitute endorsement by the U.S. Geological Survey or U.S. Army.)

Reactions of 4-Methylcatechol with Amines. Approximately 55 mg of 4ADNT, 2ADNT, 2,4DANT, or 2,6DANT were dissolved separately in 1 L pH 6 phosphate buffer with heating. 4-Methylcatechol (4MC) (340 mg) was added to the individual solutions at room temperature and allowed to stir open to the atmosphere for 16−26 days. The reaction solutions were then passed through C18 Mega-Bond Elut cartridges (Varian Chromatography Products), and the colored reaction products were eluted with methanol. Methanol was removed using a rotary evaporator, and the products were redissolved in 2−3 mL DMSO-d_6 for NMR analysis.

Blank Reactions of Diamines with HRP. Fifty milligrams of 2,4DANT or 2,6DANT was dissolved in 1 L of pH 6 phosphate buffer, charged with 100 mg of HRP and 8 mL of H_2O_2 (3% solution), and allowed to stir for 3 days. The colored reaction products were recovered on C18 Mega-Bond Elut cartridges as just described.

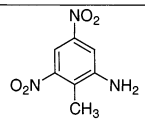
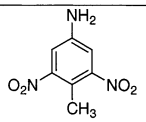
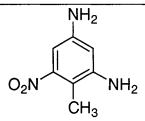
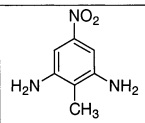
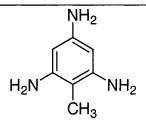
HRP Catalyzed Reactions of Coniferyl Alcohol with 4ADNT and 2,4DANT. Coniferyl alcohol (115 mg), HRP (100 mg), and hydrogen peroxide (8 mL) were added to a solution of 100 mg of 4ADNT or 200 mg of 2,4DANT dissolved in 2 L of pH 6 phosphate buffer. The colored reaction products were recovered on C18 Mega-Bond Elut cartridges as described above after 7 days of reaction.

Reactions of IHSS Soil Humic Acid with Amines. Approximately 180−200 mg of the monoamines 2ADNT or 4ADNT was dissolved in 4 L, 200 mg of the diamines 2,4DANT or 2,6DANT was dissolved in 2 L, and 150 mg of TAT was dissolved in 0.02 L of deionized and distilled water. Humic acid solutions were prepared by adjusting 500 mg of the H^+-saturated soil humic acid in 400 mL of H_2O to pH 6.4 with 1 N NaOH. For HRP catalyzed reactions, 120 mg of the enzyme was dissolved in the humic acid solution. The humic acid solutions were added to the solutions of dissolved amine, and pH was adjusted to ~6.0 if necessary. For HRP catalyzed reactions, 16 mL of hydrogen peroxide was then added to the combined solutions of amine, humic acid, and enzyme; 63 mg of birnessite was added to the reaction solution of 2,4DANT and humic acid. The solutions were stirred open to the atmosphere and at room temperature 14−24 days for the noncatalyzed and 9−13 days for the catalyzed reactions. The samples were then re-H^+-saturated by passing the solutions through a Dowex MSC-1 cation exchange column (Dow Chemical) and freeze-dried.

Sawdust. Approximately 2 g of sawdust (particle size less than 10 mm; mixture of hard- and softwood) and 75 mg of birnessite were added to a solution of 150 mg of 2,4DANT dissolved in 1.5 L of H_2O; a control reaction without birnessite was also performed. The slurries were stirred for 17 days open to the atmosphere. The sawdust was collected on a sintered glass funnel and washed with acetonitrile until free of the unreacted yellow 2,4DANT, air-dried, and then desiccated.

NMR Spectroscopy. Liquid-phase ^{15}N NMR spectra were recorded on a Varian XL300 or GEMINI 2000 NMR spec-

TABLE 1. pK_a's and ^{15}N NMR Chemical Shifts for Amino Groups of Reduced TNT Amines[a]

	 2ADNT	 4ADNT	 2,4DANT	 2,6DANT	 TAT[1]
Liquid State δ ^{15}N	70.6 ppm	66.9 ppm	62.4 ppm (2-NH_2) 59.8 ppm (4-NH_2)	62.0 ppm	58.3 ppm (2,6-NH_2) [49.9 ppm] 55.2 ppm (4-NH_2) [47.3 ppm]
Solid State δ ^{15}N	68.3 ppm	58.1 ppm	57.7 ppm	60.2 ppm	54.7 ppm
pK_a	0.36	0.95	3.13 (para) 2.58 (0.47) (ortho)	2.54 (0.74)	5.31 (para) 4.75 (2.91) (ortho)

[a] Chemical shifts reported in ammonia scale. Liquid-phase chemical shifts determined in DMSO-d_6, except for bracketed values, determined in D_2O. pK_a values from ref *17*; for the ortho amino groups, the first number refers to the intrinsic value and the number in parentheses refers to the pK_a after titration of the para group. [1]Trihydrochloride salt.

trometer at a nitrogen resonance frequency of 30.4 MHz using a 10 mm broadband probe. Spectra of the methanol soluble fractions of product mixtures from 4MC and the amines, blank reactions of 2,4DANT and 2,6DANT with HRP, and HRP catalyzed reactions of 4ADNT and 2,4DANT with coniferyl alcohol (all dissolved in 2−3 mL of DMSO-d_6) were acquired using a 18 656.7 Hz spectral window (613.7 ppm), 45° pulse angle, 0.5-s acquisition time, 5.0-s pulse delay, and inverse gated decoupling. ACOUSTIC (*23*) spectra were recorded on the reacted humic acids (~300 mg humic acid and 80 mg $Cr(Acac)_3$ dissolved in 2 mL of DMSO-d_6) as previously described (*19*). The DEPT (distortionless enhancement by polarization transfer) spectrum was acquired using a 2.0-s pulse delay for proton relaxation and $^1J_{NH}$ of 90 Hz. Neat formamide in a 5 mm NMR tube, assumed to be 112.4 ppm, was used as an external reference standard for all spectra. The ^{15}N NMR chemical shifts are reported in ppm downfield of ammonia, taken as 0.0 ppm.

Solid-state CP/MAS (cross polarization/magic angle spinning) ^{15}N NMR spectra were recorded on a Chemagnetics CMX-200 NMR spectrometer at a nitrogen resonance frequency of 20.3 MHz, using a 7.5 mm ceramic probe (zirconium pencil rotors), 30 000 Hz spectral window, and 17.051-ms acquisition time. Acquisition parameters for humic acid/2ADNT and sawdust/2,4DANT included a 5.0-ms contact time, 0.5-s pulse delay, and spinning rate of 5 kHz; 5.0-ms contact time and 5.0-s pulse delay for humic acid/2,6DANT/HRP;and 2-ms contact time and 1.0-s pulse delay for humic acid/2,4DANT. Nitrogen-15 chemical shifts were referenced to glycine, taken as 32.6 ppm.

Liquid-phase inverse gated decoupled ^{15}N NMR spectra of the model compound reaction product mixtures and ACOUSTIC ^{15}N NMR spectra of the humic acids provide quantitative distributions of the nitrogens in the samples. Solid-state CP/MAS ^{15}N NMR spectra should only be interpreted semiquantitatively because of uncertainties in the quantitative accuracy of the CP experiment (*24*).

Results

Background. Reactions of aromatic amines with organic functional groups are summarized in Figure A (Supporting Information) and ^{15}N NMR chemical shifts of model compounds corresponding to the resulting reaction products listed in Figure B (Supporting Information). In the context of this background information, an updated analysis of ^{15}N NMR spectra of Suwannee River fulvic acid reacted with aniline in aqueous solution, in organic solvent, and in aqueous solution with HRP and mushroom tyrosinase as catalyst (Figure C) is provided in the Supporting Information section, along with a summary of assignments (Table A). Details of the kinetics and pH effects in the binding of aniline with dissolved and sedimentary organic matter were reported in refs *25* and *26*. More recently, substituent effects on the binding and transformation of para-substituted anilines to soils (*27*) and the photo-Fenton reaction of aniline with peat humic acid have been reported (*28*).

Reaction of Amines with 4-Methylcatechol. To confirm the ability of the mono- and diamines to undergo nucleophilic addition reactions with quinone groups, 2ADNT, 4ADNT, 2,4DANT, and 2,6DANT were reacted with 4-methylcatechol in aqueous solution at pH 6 without catalysts. The ^{15}N NMR chemical shifts of the amino compounds along with pK_a's of the amino groups are shown in Table 1. Although the pK_a's of the monoamines would suggest that they are only weakly nucleophilic (0.36 and 0.95 for 2ADNT and 4ADNT, respectively), their ability to condense with the quinone is confirmed in the NMR spectra of the product mixtures (Figure 2). For example, 4ADNT reacts with 4MC to form a number of products, as indicated by the multiplicity of discreet peaks in the NMR spectrum. The peaks around 88 ppm correspond to aminohydroquinone nitrogens and the peaks around 106 ppm to aminoquinone nitrogens. The minor peak at 301 ppm corresponds to imine nitrogen. Several forms of nitrogen may account for the peaks from approximately 120 to 150 ppm, including carbazoles, indoles, pyrroles, amides, and quinolones. Only a few products result from the reaction of 2ADNT with 4MC, consistent with the lower pK_a and presumed weaker nucleophilicity of 2ADNT compared to 4ADNT.

The spectrum of the product mixture from the reaction of 2,4DANT and 4MC exhibits mainly broad bands, in contrast to the spectra of the monoamine reaction mixtures (Figure 2). The broad bands can be interpreted as representing a product mixture with a number of components much greater than in the monoamine mixtures. This again is consistent with the higher pK_a and greater nucleophilicity of 2,4DANT.

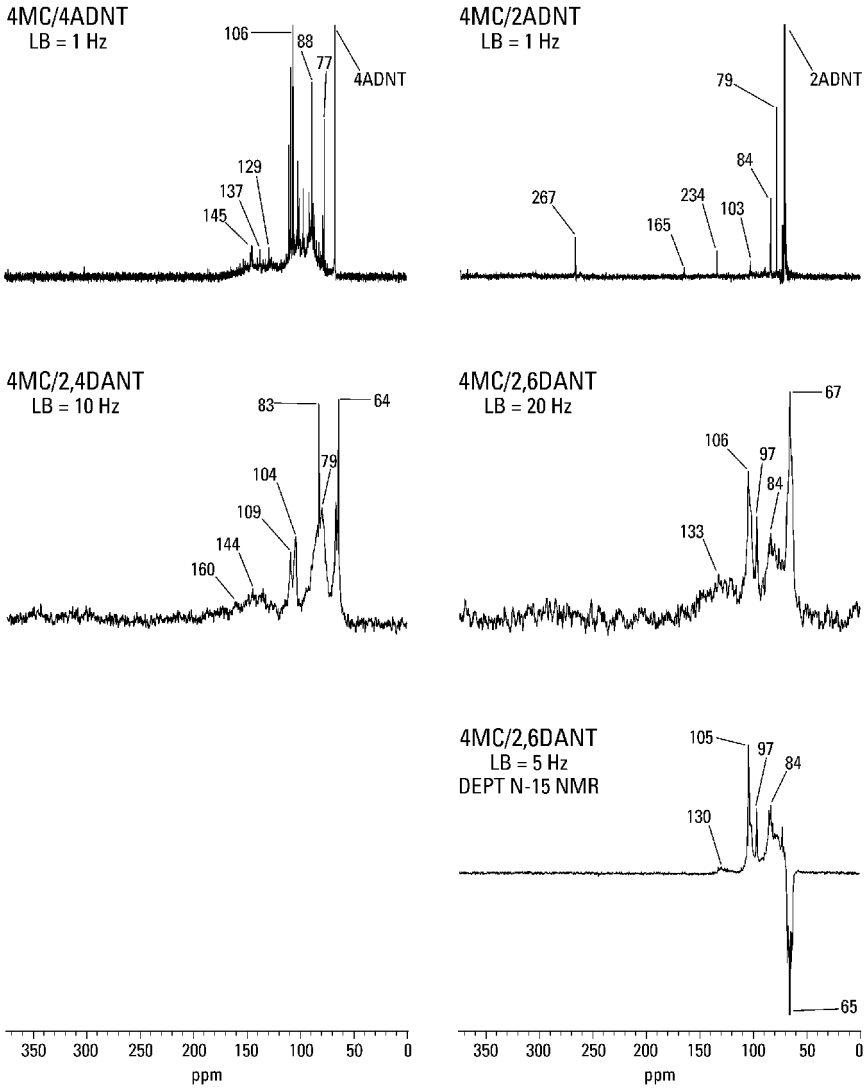
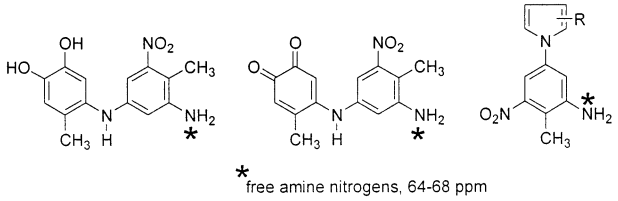


FIGURE 2. Liquid-phase ^{15}N NMR spectra (inverse gated decoupled) of product mixtures from reactions of 2ADNT, 4ADNT, 2,4DANT, and 2,6DANT with 4-methylcatechol. LB = line broadening. DEPT spectrum: nitrogens bonded to two protons inverted; nitrogens bonded to one proton in positive phase.

Because both amine positions in the 2,4DANT are labeled, which of the two amine groups has condensed with the quinone cannot be determined directly from the spectrum. A reasonable assumption is that the 2-amino group is less reactive than the 4-amino group because of steric hindrance effects. The broad peak at 64 ppm corresponds to the free amine* groups (Scheme 1) of the 2,4DANT molecules condensed with a 4MC molecule or larger oligomeric unit (trimer, tetramer, etc.). The 2,6DANT/4MC product mixture is somewhat similar to that of 2,4DANT. The peak at 67 ppm again represents the free amine groups of the 2,6DANT molecules condensed with the 4MC. This is confirmed in the DEPT spectrum, where nitrogens bonded to two protons are inverted, and nitrogens bonded to one proton are in a positive phase.

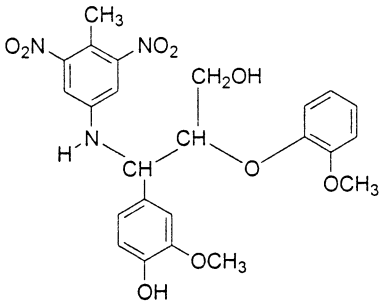
SCHEME 1



Blank Reactions of Amines with HRP. The mono- and diamines were incubated with HRP and hydrogen peroxide as controls for the HRP catalyzed reactions of the amines with coniferyl alcohol and soil humic acid. The monoamines 4ADNT and 2ADNT did not undergo self-condensation reactions when reacted with HRP. No color changes occurred upon addition of hydrogen peroxide to the solutions of the enzyme and 4ADNT or 2ADNT. The solution of 2,4DANT and HRP darkened considerably upon addition of H_2O_2, whereas the solution of 2,6DANT and HRP darkened only slightly. A number of peaks are present in the liquid-phase ^{15}N NMR spectrum of the 2,4DANT reaction blank (Figure 3): 310, 292, and 269 ppm (azoxybenzene or imine nitrogen); 153, 134,120, and 115 ppm (assignments uncertain); 82 and 79 ppm (hydrazine nitrogen). Only a few peaks are present in the spectrum of the 2,6DANT reaction blank, at 116 and 293 ppm.

Reaction of Amines with Coniferyl Alcohol. The polymerization of coniferyl alcohol by HRP is considered one of the biosynthetic pathways to the formation of lignin in woody plant tissue. As lignin derived moieties, including coniferyl alcohol, are plausible structural units within soil humic acid, and sawdust itself is used as a composting feedstock for contaminated soils, the reaction of TNT metabolites with coniferyl alcohol is of interest. Additionally, studies on the

phytoremediation of TNT have provided evidence that 4ADNT and 2ADNT form bound residues with plant tissue (*29*−*32*). A possible mechanism for the formation of these bound residues can be derived from the studies of Sandermann et al. (*33*) on the HRP catalyzed condensation of 4-chloroaniline with coniferyl alcohol. The spectrum of the HRP catalyzed reaction of 4ADNT with coniferyl alcohol exhibits a major peak at 80 ppm (Figure 3). This chemical shift is consistent with the 4ADNT analogue of the reaction product of 4-chloroaniline with coniferyl alcohol reported by Lange et al. (*34*):



An additional peak occurs at 136 ppm. The minor peak at 375 ppm corresponds to the naturally abundant ^{15}N of the 4ADNT nitro group.

The product mixture from the HRP catalyzed reaction of coniferyl alcohol with 2,4DANT is complex. In addition to the major peak at 74 ppm, signals include a broad band of resonances from approximately 100 to 160 ppm, peaks at 254 and 271 ppm, and imine or azoxy nitrogens from 310 to 350 ppm.

Reaction of Amines with Soil Humic Acid: **Monoamines.** Quantitative liquid-phase ACOUSTIC ^{15}N NMR spectra of the soil humic acid reacted with 4ADNT, 2,4DANT, 2,6DANT, and TAT are shown in Figures 4 and 5. Solid-state CP/MAS ^{15}N NMR of soil humic acid reacted with 2ADNT are shown in Figure 6. The poor signal-to-noise ratios of the liquid-state spectra of 4ADNT reacted with the soil humic acid compared to the diamine and triamine spectra is an indication of the relatively weak nucleophilicity of the monoamine (Figures 4 and 5). The ACOUSTIC spectrum of the uncatalyzed reaction of 4ADNT with soil humic acid exhibits a broad band of resonances downfield from the residual 4ADNT peak at 67 ppm to approximately 175 ppm. A previously reported INEPT spectrum (*35*) indicated that nitrogens downfield from approximately 140 ppm in the ACOUSTIC spectrum are not directly bonded to protons and therefore represent nitrogens in heterocyclic structures. Nitrogens upfield of 140 ppm can be assigned as aminohydroquinone (∼80 ppm), aminoquinone and carbazole (∼106 ppm), and amide and indole (∼130 ppm). In general, the reaction mechanisms listed to account for heterocyclic nitrogen formation upon reaction of aniline with humic substances (*19*, *20*) (Figure A, Supporting Information) would pertain to the reactions with the reduced TNT amines, substitution patterns and steric hindrance effects permitting. The HRP increases incorporation of 4ADNT into the humic acid, as indicated by the slightly improved signal-to-noise ratio in the ACOUSTIC spectrum of the enzyme catalyzed reaction. The spectrum also consists of a broad band of resonances downfield from the residual 4ADNT peak to about 185 ppm. The maximum occurs at 136 ppm, in the region of amide or indole nitrogens. The ammonia peak at 22 ppm presumably results from deamination of 4ADNT. As discussed previously (*19*) (Figure C, Supporting Information), catalysts increase the number of potential pathways for covalent binding reactions. Certain types of covalent bonds formed only with phenoloxidase enzyme or metal catalysis may

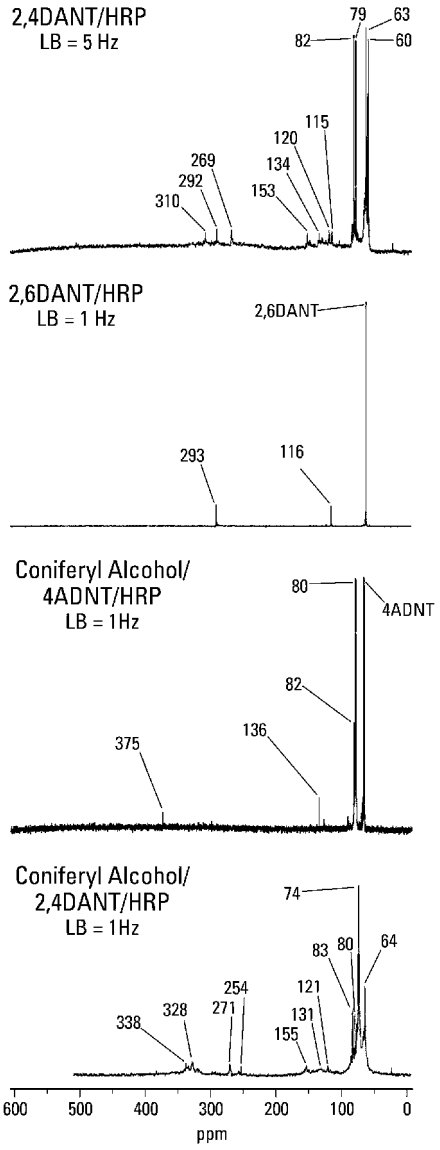


FIGURE 3. Liquid-phase ^{15}N NMR spectra (inverse gated decoupled) of product mixtures from blank reactions of 2,4DANT and 2,6DANT with HRP and HRP catalyzed reactions of 4ADNT and 2,4DANT with coniferyl alcohol. LB = line broadening.

overlap in terms of chemical shift with bonds formed in the absence of catalysts, complicating the task of assigning peaks. Thus for example, in spectra of HRP and birnessite catalyzed reactions hydrazine (∼95 ppm), arylamine (∼80 ppm), and diphenylamine (∼87 ppm) nitrogens may overlap with aminoquinone and aminohydroquinone nitrogens in the region from approximately 70−100 ppm.

The signal-to-noise ratios of the ACOUSTIC spectra of 2ADNT reacted with the soil humic acid were too weak to report, and so solid-state CP/MAS spectra are shown (Figure 6). The CP experiment suffers from limitations in resolution and quantitative accuracy, as illustrated further on. Nevertheless, the spectra clearly show that the chemical shift range of the 2ADNT nitrogens incorporated into the humic acid extends from approximately 40−220 ppm, the downfield region from ∼140 to 220 ppm confirming the occurrence of heterocyclic nitrogens. Peak maxima occur at 62, 140, and 171 ppm in the noncatalyzed reaction and 69 and 122 ppm in the HRP reaction. Additionally, some imine nitrogens are present from 300 to 350 ppm in the spectrum of the HRP-catalyzed reaction. Naturally abundant ^{15}N nuclei from the

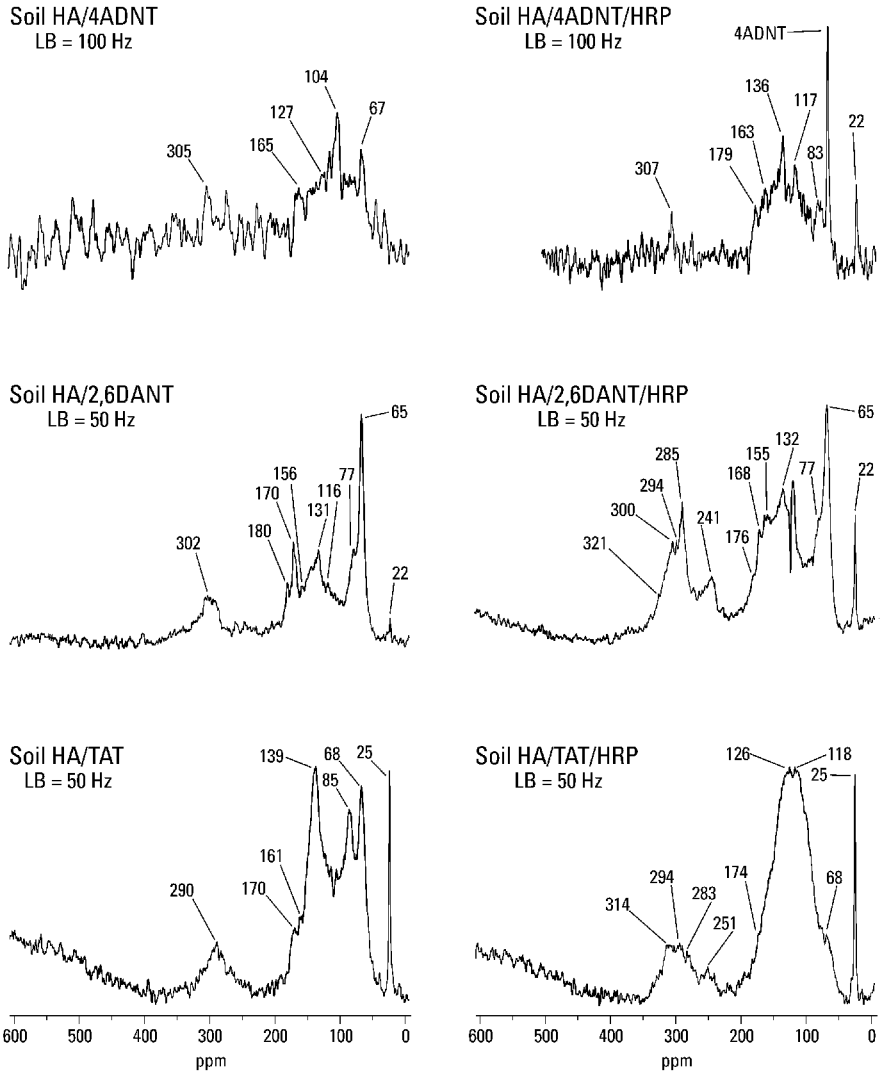


FIGURE 4. Quantitative liquid-phase ACOUSTIC ^{15}N NMR spectra of 4ADNT, 2,6DANT, and TAT reacted with IHSS soil humic acid in the presence and absence of HRP.

unlabeled nitro groups of the covalently bound 2ADNT molecules are also visible at 376 ppm.

Diamines. The ACOUSTIC spectra of the diamines reacted with the soil humic acid exhibit considerably more structural detail than the corresponding spectra of 4ADNT. The greater number of reaction products and the better signal-to-noise ratios are again an indication that the diamines are more reactive than the monoamines. In the spectrum of the uncatalyzed 2,4DANT reaction, well resolved peaks occur at 65 (free amine nitrogen; Scheme 1), 77, 111, 136, 170, 181, and 305 ppm (Figure 5). Particularly noteworthy are the heterocyclic nitrogen peaks at 170 and 181 ppm and the imine peak at 305 ppm, which comprises 15% of the nitrogen incorporated into the sample (Table 2). An obvious effect of HRP on the reaction, besides increasing the overall amount of incorporation, is that the relative amount of imine formation is increased at the expense of heterocyclic nitrogen formation. The peaks at 170 and 181 ppm in the ACOUSTIC spectrum of the nonenzyme reaction are significantly diminished in the HRP spectrum. Imine nitrogen in the HRP spectrum comprises 25% of the nitrogen in the sample, compared to 15% in the nonenzyme reaction. As noted in previous studies with aniline (*19*) (Figure C, Supporting Information), HRP catalyzes the formation of 1,4-quinone groups from 3,5-disubstituted-4-hydroxybenzene carboxylic acid moieties (e.g. syringic acid) via an oxidative decarboxylation mechanism. The 1,2-addition of 2,4DANT to these hindered 1,4-quinone groups to form stable imine adducts is apparently favored over the sequence of condensation reactions leading to the formation of heterocyclic nitrogen adducts. The very weak peak at 248 ppm may correspond to the unidentified nitrogens (imidazoles, oxazoles, pyrazoles, or nitriles) reported in the aniline study (*19*) (Figure C, Supporting Information). These are more clearly visible (247 ppm) in the spectrum of 2,4DANT reacted with soil humic acid in the presence of birnessite, which essentially replicates the effects of HRP (Figure 5). Similar to HRP, birnessite effects a shift away from heterocyclic nitrogen condensation product toward imine formation. Imines comprise 20% of the nitrogens incorporated into the sample. Both birnessite and HRP appear to increase the amount of deamination, as evident in the intensities of the ammonia peaks at 22 ppm.

The uncatalyzed reaction of 2,6DANT with soil humic acid results in a distribution of condensation products similar to 2,4DANT, with peaks at 65, 77, 116, 131, 156, 170, 180, and 302 ppm (Figure 4). Free amine, aminohydroquinone, aminoquinone, amide, heterocyclic, and imine nitrogens are all present. The effects of HRP on the reaction of 2,4DANT with the humic acid are also replicated in the case of 2,6DANT. Imine formation is increased at the expense of heterocyclic nitrogen formation. The heterocyclic nitrogen peaks at 170 and 180 ppm in the spectrum of the noncatalyzed reaction are diminished in the HRP-catalyzed reaction. Imines comprise 16% of the nitrogens in the noncatalyzed

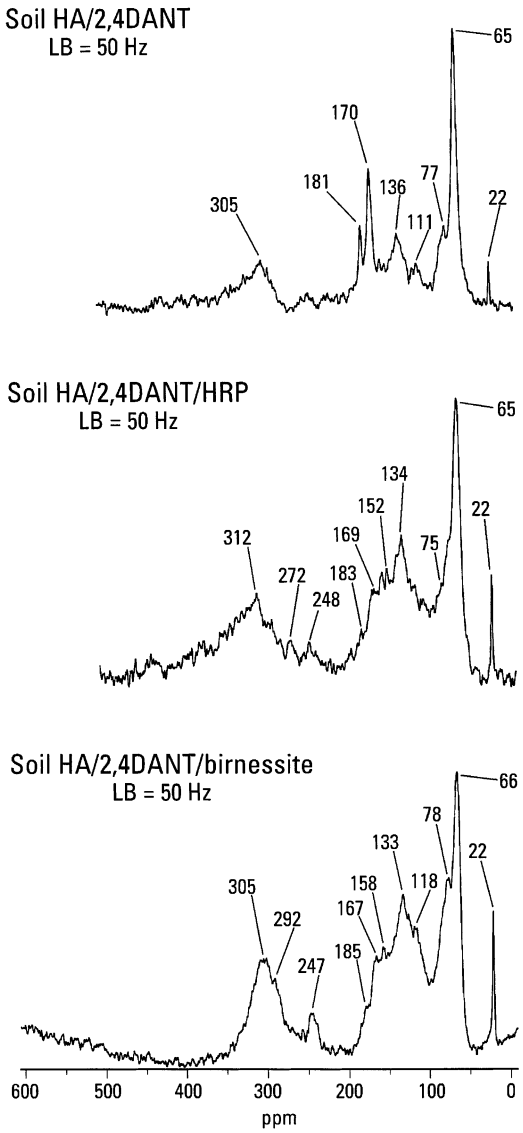


FIGURE 5. Quantitative liquid-phase ACOUSTIC ^{15}N NMR spectra of 2,4DANT reacted with IHSS soil humic acid: uncatalyzed; HRP catalyzed; birnessite catalyzed.

reaction and 21% in the HRP-catalyzed reaction. Nitrogens diagnostic of catalysis are present at 241 ppm. HRP also increases the release of ammonia via deamination.

The peak areas of the free amine nitrogens (50−70 ppm; Scheme 1) listed in Table 2 suggest that some of the 2,4- and 2,6DANT molecules are covalently bound to humic acid through both amino groups. If only one amine group of each 2,4- or 2,6DANT molecule were covalently bound to humic acid, the free amine peak from 50 to 70 ppm would comprise 50% of the total spectrum area. The peak areas range from 13% to 30%. Interestingly, HRP and birnessite appear to increase the proportion of both 2,4DANT and 2,6DANT molecules bound to the humic acid through both amine groups.

Triaminotoluene. The trihydrochloride salt of TAT was the reagent used in reactions with soil humic acid. Although the trihydrochloride salt is stable in the solid state, once dissolved in water and exposed to atmospheric oxygen, the TAT molecule becomes unstable and slowly begins to degrade. Hawari et al. (*36*) reported hydrolysis of TAT into hydroxydiaminotoluene (4-hydroxy-2,6-diaminotoluene and 2-hydroxy-4,6-diaminotoluene) and dihydroxyaminotoluene (2,4-dihydroxy-6-aminotoluene) under aerated conditions, with eventual formation of a precipitate due to polymerization of the degradation products. Hydrolysis was enhanced by acidification (pH 2−3) and/or heating with water (*36*). Here the ^{15}N NMR spectra (Figure 4) are interpreted based upon the assumption that reaction of TAT with the humic acid in solution occurs at a faster rate than the degradation of TAT and that the majority of nitrogens observed in the spectra represent TAT covalently bonded to the humic acid and not degradation products of TAT. The presence of ammonia peaks at 25 ppm in the spectra, however, indicates that some hydrolysis of the TAT molecules occurred. Consistent with the presumed strength of TAT as a nucleophile, the ACOUSTIC spectra of TAT reacted with soil humic acid (Figure 4) have signal-to-noise ratios and resolution comparable to those of the diamines reacted with the humic acid. The ACOUSTIC spectrum of the uncatalyzed reaction has well resolved peaks at 68, 85, 139, 161, 170, and 290 ppm. Its quantitative distribution of nitrogens differs somewhat from the comparable spectra of 2,4DANT and 2,6DANT. The dominant peak in the uncatalyzed TAT spectrum occurs at 139 ppm, in the region of protonated heterocyclic or amide nitrogens, while the nonprotonated heterocyclic nitrogen peaks at 161 and 170 ppm are diminished. The amount of imine formation is significant (9%) but less than in the uncatalyzed reactions of the diamines. The effect of HRP on the reaction of TAT with the humic acid replicates the results observed with the diamines. A shift away from heterocyclic toward imine nitrogen formation is evident, the imines comprising 13% of all nitrogens. Formation of the unidentified nitrogens (251 ppm) is also observed. In both the uncatalyzed and HRP catalyzed reactions, formation of ammonia from deamination of either the bound or unbound TAT is again evident. If each TAT molecule were covalently bonded to the humic acid through only one amine group, then the free amine nitrogens (68 ppm) would comprise 66% of the total nitrogens in the spectra. The fact that the free amine nitrogens comprise 5−16% of the total nitrogens in the spectra (Table 2) suggests binding of the TAT molecule to humic acid occurs through two or all three amine groups in some cases.

Comparison of Solid- and Liquid-State Spectra. As background for an examination of the composting of TNT contaminated soil using solid-state NMR, comparison of the ACOUSTIC and solid-state CP/MAS ^{15}N NMR spectra of the 2,4DANT/SHA and 2,6DANT/SHA/HRP reactions is instructive (Figure 6). Significant differences in resolution between the solid- and liquid-state spectra as well as discrepancies in the quantitative distribution of nitrogens are apparent. For example, the well resolved heterocyclic nitrogen peaks at 170 and 181 ppm in the ACOUSTIC spectrum of 2,4DANT/SHA (Figure 5) are poorly resolved and diminished in intensity in the CP/MAS spectrum (Figure 6). Overall, the heterocyclic and imine nitrogens are underestimated quantitatively in the CP/MAS spectrum. The loss in resolution is even more dramatic in going from the liquid- to solid-state spectrum of the 2,6DANT/SHA/HRP reaction. The distinct peaks at 176, 168, 155, and 132 ppm in the ACOUSTIC spectrum (Figure 4) are completely broadened out in the CP/MAS spectrum (Figure 6). The peak at 241 ppm in the ACOUSTIC spectrum, diagnostic of enzyme or metal catalysis, is poorly resolved and below the signal-to-noise level of the spinning sidebands in the CP/MAS spectrum. The structural detail within the imine region of the ACOUSTIC spectrum is absent in the CP/MAS spectrum. The imine and heterocyclic nitrogens are also significantly underestimated quantitatively in the CP/MAS spectrum of the 2,6DANT/SHA/HRP reaction.

Reaction of 2,4DANT with Sawdust. In reactions discussed thus far, the soil humic acid and model compounds were completely dissolved in solution. Experiments were also

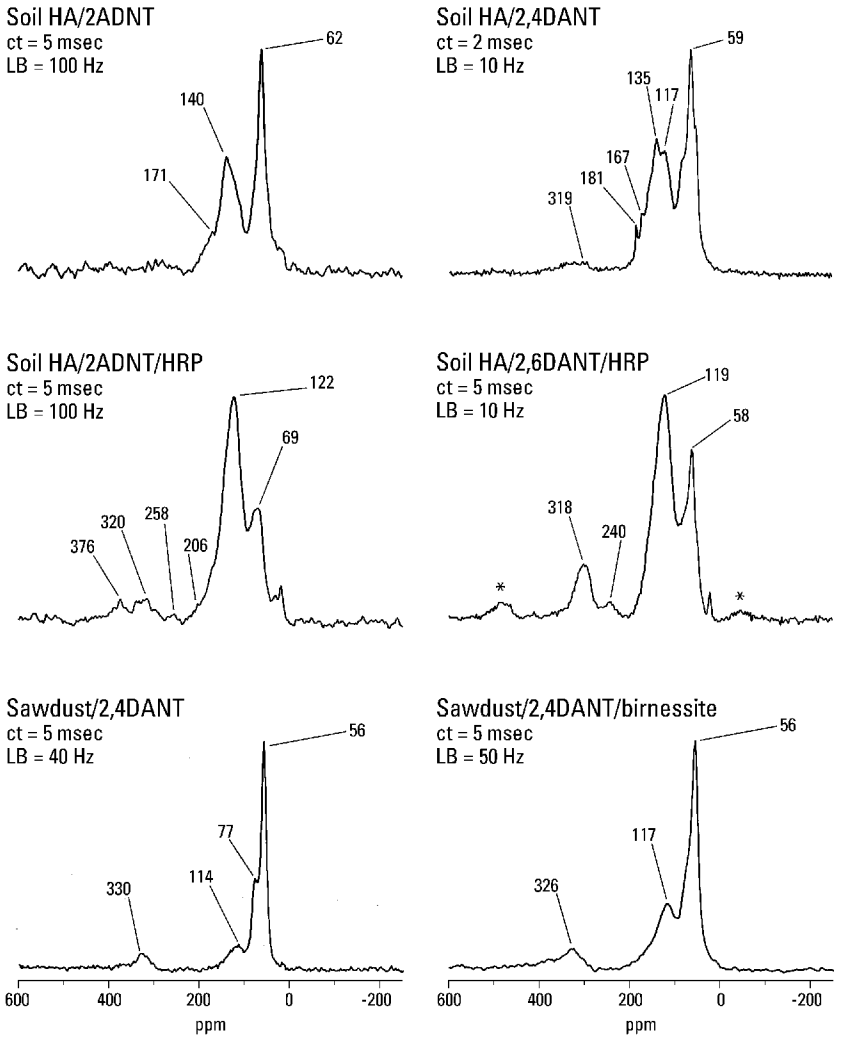


FIGURE 6. Solid-state CP/MAS ^{15}N NMR spectra of 2ADNT, 2,4DANT, and 2,6DANT reacted with IHSS soil humic acid and 2,4DANT reacted with sawdust (lignocellulose). HRP = horseradish peroxidase; ct = contact time. LB = line broadening. Asterisks indicate spinning sidebands.

TABLE 2. Peak Areas as Percent of Total Nitrogen for Quantitative Liquid-Phase ACOUSTIC ^{15}N NMR Spectra of IHSS Soil Humic Acid Reacted with Reduced TNT Amines[a]

	350−375 ppm imine	275−200 ppm imidazole, oxazole, pyrazole, nitrile	200−140 ppm heterocyclic	140−70 ppm amide, carbazole, aminoquinone, aminohydroquinone	70−50 ppm free amine
4ADNT	0	0	27	73	0
4ADNT/HRP	4	0	38	58	0
2,4DANT	15	0	26	28	30
2,4DANT/HRP	25	6	20	31	18
2,4DANT/birn	20	8	20	38	13
2,6DANT	16	0	26	35	23
2,6DANT/HRP	21	12	21	30	16
TAT	9	4	21	50	16
TAT/HRP	13	7	22	54	5

[a] Electronic integration.

performed where the dissolved amines were allowed to react with insoluble whole peat or sawdust in an aqueous slurry. The whole peat results have been reported (*35*). Sawdust has been used as an amendment in laboratory scale aerobic composting experiments designed to mimic large scale windrow composting of TNT contaminated soils (*16*, *21*, *37*). In these experiments, the majority of the solvent nonextractable TNT-derived ^{14}C label became incorporated into the lignocellulose fraction of the finished compost. Woodchips are often used as bulking agents in large scale windrow composting (*8*). Solid-state CP/MAS ^{15}N NMR spectra confirm covalent binding of 2,4DANT to the sawdust (Figure 6). The oxygen containing functional groups of the lignin portion of the lignocellulose are the most likely substrate sites for binding by the 2,4DANT. Within the limits of resolution and quantitation of the CP experiment just discussed, the spectra of the sawdust suggest a distribution of covalent bonds somewhat similar to that between 2,4DANT and humic acid

reported here and between 2,4DANT and peat (*35*). In the spectrum of the birnessite catalyzed reaction, peak maxima occur at 56 ppm (free amine nitrogens), 117 ppm (aminoquinone nitrogens), and 326 ppm (imine nitrogens). The broad envelope of nitrogens from ~200 to 70 ppm would also encompass heterocyclic and aminohydroquinone nitrogens as well as the arylamine nitrogens at about 74−80 ppm observed in the reaction of 4ADNT and 2,4DANT with coniferyl alcohol. Without birnessite, the amount of covalent binding appears less, judging by the signal-to-noise ratio of the spectrum. Peak maxima occur at 330 ppm (imine), 114 ppm (aminoquinone), 77 ppm (aminohydroquinone), and 56 ppm (free amine).

Discussion

The NMR studies have confirmed the ability of the monoaminodinitrotoluene, diaminonitrotoluene, and triaminotoluene reductive degradation products of TNT to form covalent bonds with soil humic acid. Binding of the diamines to lignocellulose has also been confirmed. The reactivity of the reduced TNT amines with humic substances mirrors the reactivity of aniline in several respects. All the reduced TNT amines undergo nucleophilic addition with quinone and other carbonyl groups to form both heterocyclic and nonheterocylic condensation products. On the other hand, the diaminonitrotoluenes and TAT undergo 1,2 addition with quinone groups in soil humic acid to form imines to a much greater extent than do aniline, 2ADNT, and 4ADNT. An important consideration is that the nucleophilic addition reactions occur without enzyme or metal catalysis. HRP increased the incorporation of all the reduced TNT amines into soil humic acid, as was the case with aniline. Two important features observed in the HRP and birnessite catalyzed reactions of aniline with humic substances were also observed in the reactions with 2,4DANT, 2,6DANT, and TAT: an increase in the amount of imine formation over the noncatalyzed reaction; and formation of the nitrogens at around 241−251 ppm (imidazole, oxazole, pyrazole). These two effects of catalysis were observed to a lesser degree with the 2ADNT; the signal-to-noise levels of the 4ADNT spectra were too low to make this conclusion.

The reactions of the reduced TNT amines with humic acid were performed in the context of confirming the covalent binding of the TNT metabolites with organic matter during aerobic composting of contaminated soils. These reactions, however, would be relevant to other bioremediation matrices (*38*, *39*) as well as to natural attenuation environments, depending on the source of organic matter, the pH, E_h, and dissolved oxygen concentrations. The confirmation of quinone groups in humic acid as sites for nucleophilic addition by the reduced TNT amines is consistent with observations of other studies concerning the effects of redox conditions on irreversible binding of the amines to organic matter. Elovitz and Weber (*17*) reported that 2,4DANT demonstrated a stronger affinity for irreversible binding to organic matter in oxic sediments than in anoxic sediments and suggested that under reducing conditions the quinone moieties are reduced (or partially reduced) and therefore unavailable for nucleophilic attack by the amine. In other words, if redox conditions are poised such that the redox equilibrium favors the reduced hydroquinone form, nucleophilic addition of the aromatic amine is precluded. Maintaining oxygenated conditions to maximize the availability of quinone groups for condensation with the aromatic amines is a partial explanation behind the recommendation of several groups for two stage anaerobic/aerobic treatment of contaminated soils (*12*, *40*, *41*). Reduction of nitro groups is favored under anaerobic conditions; covalent binding of the corresponding aromatic amines is favored under oxic conditions. However, in the absence of oxygen numerous pathways still exist for condensation of aromatic amines with nonquinoidal carbonyl groups in organic matter to form heterocyclic condensation products.

The production and fate of TAT during biological treatments of contaminated soils and under conditions of natural attenuation has been a matter of uncertainty, due in part to the chemical instability of the TAT molecule under oxic conditions and the resulting analytical difficulties in measuring its concentration. Kinetic evidence for irreversible binding of TAT to humic acid in laboratory experiments has been reported (*42*). Complete reduction of TNT to TAT has been documented in iron reducing aquifer columns supplemented with acetate, under which conditions the TAT remained stable (*13*). Reduction of TNT to TAT has also been carefully documented in an anaerobic sludge (*36*). There do not appear to be any reports on the formation or detection of TAT during composting or of the hydrolytic release of TAT from finished composts. One could speculate that in composting situations where the strictly anaerobic conditions for complete reduction of TNT to TAT are met, the large reservoir of organic matter in the compost could provide a sink for instantaneous covalent binding by the TAT. Confirmation of the facile covalent binding of TAT with the soil humic acid is consistent with this hypothesis.

Thorne and Leggett measured solvent extractable and hydrolyzable TNT metabolites in time course experiments of composting Umatilla soils (*43*, *44*). They observed that the transformation of TNT to solvent nonextractable transformation products went through two stages of covalent conjugation in compost. In the first stage, about 20% of the transformed TNT could be released through hydrolysis. As processing continued, a second stage occurred in which the bonds were either altered to form different functional groups that were not hydrolyzable, or additional bonds formed as the bound transformation products were further reduced and conjugated through multiple bonds. Two results from the NMR studies may provide a partial explanation for these observations, namely, confirmation of the ability of the reduced TNT amines to form heterocyclic condensation products with soil organic matter, and evidence that 2,4DANT, 2,6DANT, and TAT undergo covalent binding to organic matter through more than one amine group. Examples of inter- and intramolecular condensation reactions of aromatic amines with functional groups resulting in heterocyclic nitrogen formation were discussed in previous papers (*19*, *20*) and summarized in Figure A (Supporting Information). Initial bond formation in these multistep reactions could account for the first stage of 20% hydrolyzability during composting, whereas final condensation to the heterocycle could account for the second stage of nonhydrolyzability. NMR evidence for binding through two amine groups is consistent with the suggestion that the second stage of nonhydrolyzability could result from covalent binding of the TNT metabolites through multiple bonds.

Acknowledgments

This study was funded through the Department of Defense Strategic Environmental Research and Development Program, Arlington, VA. Helpful discussions with Judith Pennington, Phil Thorne, and Tom Jenkins, U.S. Army Corps of Engineers, Ron Spanggord, Stanford Research Institute, and Paul Gates, USGS, are appreciated.

Supporting Information Available

Summary of assignments for ^{15}N NMR spectra of humic substances reacted with aniline (Table A), reactions of aromatic amines with organic functional groups (Figure A), and ^{15}N NMR chemical shifts of model compounds relevant to this study (Figure B) and an updated analysis of ^{15}N NMR

spectra of Suwannee River fulvic acid reacted with aniline in aqueous solution, in organic solvent, and in aqueous solution with HRP and mushroom tyrosinase (Figure C). This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Spain, J. C. *Biodegradation of Nitroaromatic Compounds*; Plenum Press: New York, 1995.

(2) Alleman, B. C.; Leeson, A. *Bioremediation*; Battelle Press: Columbus, 1997; Vol. 4(2).

(3) Alleman, B. C.; Leeson, A. *The Fifth International In Situ and On-Site Bioremediation Symposium*; Battelle Press: Columbus, 1999; Vol. 5(7).

(4) Spain, J. C.; Hughes, J. B.; Knackmuss, H.-J. *Biodegradation of Nitroaromatic Compounds and Explosives*; Lewis Publishers: Boca Raton, 2000.

(5) Harvey, S. D.; Fellows, R. J.; Cataldo, R. A.; Bean, R. M. *Environ. Toxicol. Chem.* **1991**, *10*, 845−855.

(6) Macdonald, J. A. *Environ. Sci. Technol.* **2001**, *35*(17), 372A−376A.

(7) Jenkins, T. F.; Walsh, M. E.; Thorne, P. G.; Thiboutot, S.; Ampleman, G.; Ranney, J. A.; Grant, C. A. Special Report CCREL-SR-97-22; U.S. Army, Cold Regions Research and Engineering Laboratory: Hanover, NH, 1997.

(8) Jerger, D. E.; Woodhull, P. M. In *Biodegradation of Nitroaromatic Compounds and Explosives*; Spain, J. C., Hughes, J. B., Knackmuss, H. J., Eds.; Lewis Publishers: Boca Raton, 2000; pp 395−423.

(9) *Remediation Case Studies: Bioremediation and Vitrification;* EPA 542R97008; 1997; Vol. 5, pp 107−153.

(10) Remediation Technologies Screening Matrix and Reference Guide, Version 4.0; Federal Remediation Technologies Roundtable. http://www.frtr.gov/matrix2/section3/Table3_7_nfr.html (accessed May 2002).

(11) Haderlein, S. B.; Schwarzenbach, R. P. In *Biodegradation of Nitroaromatic Compounds*; Spain, J. C., Ed.; Plenum Press: New York, 1995; pp 199−225.

(12) Rieger, P.-G.; Knackmuss, H. J. In *Biodegradation of Nitroaromatic Compounds*; Spain, J. C., Ed.; Plenum Press: New York, 1995; pp 1−18.

(13) Hofstetter, T. B.; Heijman, C. G.; Haderlein, S. B.; Holliger, C.; Schwarzenbach, R. P. *Environ. Sci. Technol.* **1999**, *33*, 1479−1487.

(14) Preuss, A.; Rieger, P.-G. In *Biodegradation of Nitroaromatic Compounds*; Spain, J. C., Ed.; Plenum Press: New York, 1995; pp 69−85.

(15) Rugge, K.; Hofstetter, T. B.; Haderlein, S. B.; Bjerg, P. L.; Knudsen, S.; Zraunig, C.; Mosbaek, H.; Christensen, T. H. *Environ. Sci. Technol.* **1998**, *32*, 23−31.

(16) Pennington, J. C.; Hayes, C. A.; Myers, K. F.; Ochman, M.; Gunnison, D.; Felt, D. R.; McCormick, E. F. *Chemosphere* **1995**, *30*, 429−438.

(17) Elovitz, M. S.; Weber, E. J. *Environ. Sci. Technol.* **1999**, *33*, 2617−2625.

(18) Thorn, K. A. *Division of Environmental Chemistry Preprints of Papers*; 213th National Meeting of the American Chemical Society: San Francisco, CA, 1997; pp 305−306.

(19) Thorn, K. A.; Goldenberg, W. S.; Younger, S. J.; Weber, E. J. In *Humic and Fulvic Acids: Isolation, Structure, and Environmental Role*; Gaffney, J. S., Marley, N. A., Clark, S. B., Eds.; American Chemical Society: 1996; pp 299−326.

(20) Thorn, K. A.; Pettigrew, P. J.; Goldenberg, W. S.; Weber, E. J. *Environ. Sci. Technol.* **1996**, *30*, 2764−2775.

(21) Thorn, K. A.; Pennington, J. C.; Hayes, C. A. *Environ. Sci. Technol.* **2002**, *36*, 3797−3805.

(22) Spanggord, R. J. *J. Labeled Compds. Radiopharm.* **1998**, *41*, 615−621.

(23) Patt, S. L. *J. Magn. Reson.* **1982**, *49*, 161−163.

(24) Stejskal, E. O.; Memory, J. D. *High-Resolution NMR in the Solid State: Fundamentals of CP/MAS*; Oxford University Press: New York, 1994.

(25) Weber, E. J.; Spidle, D. J.; Thorn, K. A. *Environ. Sci. Technol.* **1996**, *30*, 2755−2763.

(26) Weber, E. J.; Colon, D.; Baughman, G. L. *Environ. Sci. Technol.* **2001**, *35*, 2470−2475.

(27) Li, H.; Lee, L. S.; Jafvert, C. T. *Environ. Sci. Technol.* **2000**, *34*, 3674−80.

(28) Fukushima, M.; Tatsumi, K.; Morimoto, K. *Environ. Sci. Technol.* **2000**, *34*, 2006−2013.

(29) Bhadra, R.; Wayment, D. G.; Hughes, J.; Shanks, J. *Environ. Sci. Technol.* **1999**, *33*, 446−452.

(30) Palazzo, A. J.; Leggett, D. C. *J. Environ. Qual.* **1986**, *15*, 49−52.

(31) Thompson, P. L.; Ramer, L. A.; Schnoor, J. L. *Environ. Sci. Technol.* **1998**, *32*, 975−980.

(32) Thorne, P. G. Special Report 99-19; U.S. Army Corps of Engineers Cold Regions Research & Engineering Laboratory: Hanover, NH, 1999.

(33) von der Trenck, K. T.; Hunkler, D.; Sandermann, H. *Z. Naturforsch.* **1981**, *36*, 714−720.

(34) Lange, B. M.; Hertkorn, N.; Sandermann, J. H. *Environ. Sci. Technol.* **1998**, *32*, 2113-2118.

(35) Thorn, K. A. In *Explosives Conjugation Products in Remediation Matrices: Interim Report 2*; Pennington, J. C., Thorn, K. A., Gunnison, D., McFarland, V. A., Thorne, P. G., Inouye, L. S., Fredrickson, H., Leggett, D. C., Ringleberg, D., Jarvis, A. S., Felt, D. R., Lutz, C. H., Hayes, C. A., Clarke, J. U., Richmond, M., O'Neal, B., Porter, B. E., Eds.; Technical Report SERDP-98-12; U.S. Army Engineer Waterways Experiment Station: Vicksburg, MS, 1998; pp 7−37.

(36) Hawari, J.; Halasz, A.; Paquet, L.; Zhou, E.; Spencer, B.; Ampleman, G.; Thiboutot, S. *Appl. Environ. Microbiol.* **1998**, *64*, 2200−2206.

(37) Pennington, J. C.; Thorn, K. A.; Inouye, L. S.; McFarland, V. A.; Jarvis, A. S.; Lutz, C. H.; Hayes, C. A.; Porter, B. E. *Explosives conjugation products in remediation matrices: Final report*; U.S. Army Engineer Research and Development Center: Vicksburg, MS, 1999.

(38) Achtnich, C.; Fernandes, E.; Bollag, J.-M. *Environ. Sci. Technol.* **1999**, *33*, 4448−4456.

(39) Bruns-Nagel, D.; Knicker, H.; Drzyzga, O. *Environ. Sci. Technol.* **2000**, *34*, 1549−1556.

(40) Lenke, H.; Warrelmann, J.; Daun, G.; Walter, U.; Sieglen, U.; Knackmuss, H. J.; Alleman, B. C.; Leeson, A. In *In Situ and On-Site Bioremediation;* Alleman, B. C., Leeson, A., Eds.; Battelle Press: Columbus, OH, 1997; Vol. 2, pp 1−2.

(41) Drzyzga, O.; Bruns-Nagel, D.; Gorontzy, T.; Blotevogel, K.-H.; Gemsa, D.; von Loew, E. *Environ. Sci. Technol.* **1998**, *32*, 3529−3535.

(42) Daun, G.; Lenke, H.; Reuss, M.; Knackmuss, H.-J. *Environ. Sci. Technol.* **1998**, *32*, 1956−1963.

(43) Thorne, P. G.; Leggett, D. C. *Environ. Toxicol. Chem.* **1997**, *16*, 1132−1134.

(44) Thorne, P. G.; Leggett, D. C. In *Explosives Conjugation Products in Remediation Matrices: Interim Report 2*; Technical Report SERDP-98-12; Pennington, J. C., Thorn, K. A., Gunnison, D., McFarland, V. A., Thorne, P. G., Inouye, L. S., Fredrickson, H., Leggett, D. C., Ringleberg, D., Jarvis, A. S., Felt, D. R., Lutz, C. H., Hayes, C. A., Clarke, J. U., Richmond, M., O'Neal, B., Porter, B. E., Eds.; U.S. Army Engineer Waterways Experiment Station: Vicksburg, MS, 1998; pp 38−52.

Received for review October 24, 2001. Revised manuscript received June 26, 2002. Accepted July 2, 2002.

ES011383J